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# Human Immunodeficiency Virus Infection Alters Tumor Necrosis Factor Alpha Production via Toll-Like Receptor-Dependent Pathways in Alveolar Macrophages and U1 Cells<sup>∇</sup>

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**Human immunodeficiency virus (HIV)-positive persons are predisposed to pulmonary infections, even after receiving effective highly active antiretroviral therapy. The reasons for this are unclear but may involve changes in innate immune function. HIV type 1 infection of macrophages impairs effector functions, including cytokine production. We observed decreased constitutive tumor necrosis factor alpha (TNF- $\alpha$ ) concentrations and increased soluble tumor necrosis factor receptor type II (sTNFRII) in bronchoalveolar lavage fluid samples from HIV-positive subjects compared to healthy controls. Moreover, net proinflammatory TNF- $\alpha$  activity, as measured by the TNF- $\alpha$ /sTNFRII ratio, decreased as HIV-related disease progressed, as manifested by decreasing CD4 cell count and increasing HIV RNA (viral load). Since TNF- $\alpha$  is an important component of the innate immune system and is produced upon activation of Toll-like receptor (TLR) pathways, we hypothesized that the mechanism associated with deficient TNF- $\alpha$  production in the lung involved altered TLR expression or a deficit in the TLR signaling cascade. We found decreased Toll-like receptor 1 (TLR1) and TLR4 surface expression in HIV-infected U1 monocytic cells compared to the uninfected parental U937 cell line and decreased TLR message in alveolar macrophages (AMs) from HIV-positive subjects. In addition, stimulation with TLR1/2 ligand (Pam<sub>3</sub>Cys) or TLR4 ligand (lipopolysaccharide) resulted in decreased intracellular phosphorylated extracellular signal-regulated kinase and subsequent decreased transcription and expression of TNF- $\alpha$  in U1 cells compared to U937 cells. AMs from HIV-positive subjects also showed decreased TNF- $\alpha$  production in response to these TLR2 and TLR4 ligands. We postulate that HIV infection alters expression of TLRs with subsequent changes in mitogen-activated protein kinase signaling and cytokine production that ultimately leads to deficiencies of innate immune responses that predispose HIV-positive subjects to infection.**

Pulmonary infections are a major cause of morbidity and mortality during human immunodeficiency virus type 1 (HIV-1) infection (9). In the lung, alveolar macrophages (AMs) are the first line of defense against pathogens through protective innate immune effector functions, including enhanced microbicidal activity, phagocytosis, and induction of the proinflammatory cytokine tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ). AMs can be infected by HIV-1; they express the CD4 and CCR5 coreceptor and are targets for R5 strains of virus (39). Infection with HIV-1 leads to impaired effector functions, including alterations in cytokine production (19), which may play a role in the development of opportunistic infections (OIs). Highly active antiretroviral therapy (HAART) has led to a decrease in the incidence of OIs; however, viral reservoirs persist, and even patients successfully treated with HAART, along with those failing HAART because of nonadherence or development of drug resistance, are still susceptible to pulmonary infections, such as tuberculosis, *Streptococcus pneumoniae* pneumonia, and other pulmonary OIs.

Pathogens associated with pulmonary infections activate in-

nate immune responses through interactions with Toll-like receptor 1 (TLR1), TLR2, TLR4, and TLR6 (26). TLRs are part of a family of receptors defined by the presence of multiple leucine-rich repeats in the extracellular domain and an intracellular Toll/interleukin-1 receptor domain; to date, 13 TLRs have been identified in a variety of cells including monocytes and macrophages (10, 12). TLR2 forms heterodimers with other TLRs (e.g., TLR1 or TLR6) to recognize a broad spectrum of pathogen-associated molecular patterns that are associated with gram-positive bacteria, fungi, and mycobacteria (30, 36). TLR4 functions as a receptor for gram-negative bacterial lipopolysaccharide (LPS) and signals through the coordinated activity of several proteins, including lipid-binding protein, CD14, and MD2. Receptor engagement triggers complex TLR- and pathogen-associated molecular pattern-specific signaling cascades. These involve recruitment of adaptor molecules, such as MyD88 to the Toll/interleukin-1 receptor domain, serine kinase (IRAK) recruitment and phosphorylation, intracellular signaling via mitogen-activated protein (MAP) kinases, subsequent nuclear translocation of transcription factors, such as NF- $\kappa$ B, and upregulation of immediate-early markers of innate inflammatory responses.

TNF- $\alpha$  is a key TLR-induced inflammatory cytokine secreted in the lung by AMs. The importance of TNF- $\alpha$  in lung host immunity is highlighted by increased pulmonary infec-

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tions, usually involving *Mycobacterium tuberculosis* (7) and bacterial pathogens, in patients treated with anti-TNF- $\alpha$  drugs, such as infliximab and etanercept (8, 38). Constitutive concentrations of TNF- $\alpha$  in the lungs of HIV-1-infected individuals or from AMs exposed *ex vivo* to bacterial ligands may vary based on the stage of disease or presence of OIs (17, 33). In this study, we examined asymptomatic HIV-positive subjects on stable HAART, HIV-positive individuals on stable HAART who were failing therapy (based on CD4 and viral load measurements), and healthy HIV-negative control subjects. We observed decreased constitutive TNF- $\alpha$  in bronchoalveolar lavage fluid (BALF) samples from HIV-positive subjects compared to HIV-negative controls. This was most pronounced in HIV-positive subjects with the lowest CD4 cell counts and the highest HIV RNA (viral load). We also observed that HIV-1-infected U1 monoblastoid cells, differentiated with phorbol 12-myristate 13-acetate (PMA) to produce an AM-like phenotype, showed deficient TNF- $\alpha$  production in response to LPS (the gram-negative bacterial TLR4 ligand) and Pam<sub>3</sub>Cys (the synthetic TLR1/TLR2 ligand) compared to uninfected U937 cells. This was associated with decreased TLR surface expression and changes in the signaling pathways involved in the secretion of TNF- $\alpha$ .

## MATERIALS AND METHODS

**Study subjects.** The study was approved by the Institutional Review Boards of Boston Medical Center, New England Medical Center, and Beth Israel Deaconess Medical Center. All subjects were volunteers who gave informed consent before participating in the study. We used a comprehensive questionnaire regarding age, sex, smoking status, medical history, including HIV-1 infection, related infections, and medications. All subjects had no active pulmonary disease and normal spirometry. We obtained HIV risk factor information, peripheral blood CD4 count, HIV RNA (viral load) in plasma, history of HIV-related diseases, and antiretroviral therapy for the HIV-positive subjects who underwent venipuncture and BAL. All HIV-positive subjects were receiving HAART (stable HAART for at least the past 8 months).

**BALF sample collection and processing.** BALF samples and AMs were obtained from subjects by BAL using standard technique (20). All BALF measurements were normalized using the urea correction method, and the data were expressed as picogram of cytokine or cytokine receptor per milliliter of BALF sample. The molar ratios of TNF- $\alpha$  to soluble tumor necrosis factor receptor type II (sTNFR<sub>II</sub>) (p75/CD120b) assumed that three TNF- $\alpha$  molecules bind to three sTNFR<sub>II</sub> molecules. AMs were separated from the pooled BALF samples by centrifugation at  $100 \times g$  for 10 min at 4°C and washed in cold RPMI 1640 (GIBCO Invitrogen Cell Culture, Carlsbad, CA) supplemented with penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml) (Sigma, St. Louis, MO). Cells contained 85% to 90% AMs, 10% to 15% lymphocytes, and <1% neutrophils. Cells demonstrated >98% viability as determined by trypan blue exclusion, were >95% phagocytic of 1.1- $\mu$ m latex beads, and >95% stained positive for non-specific esterase.

**Culture of cells.** BAL cells retrieved from HIV-positive subjects and HIV-negative healthy volunteers were transferred to 24-well tissue culture plates (Falcon, Oxnard, CA) at a concentration of  $5 \times 10^5$  cells/ml in RPMI 1640 supplemented with penicillin-streptomycin (100 U/ml and 100  $\mu$ g/ml, respectively), amphotericin (0.25  $\mu$ g/ml), L-glutamine (2 mM), HEPES (2 mM; GIBCO Invitrogen Cell Culture), and 10% fetal calf serum (HyClone, Logan, UT). Cells were cultured for 16 h; nonadherent cells and supernatant fluids were removed, and fresh medium was added prior to treatment. The U937 cell line was obtained from the American Type Culture Collection (ATCC). The U1 cell line (containing two proviral copies of HIV-1) was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Thomas Folks (13). Both U937 and U1 cells were cultured in complete RPMI 1640 supplemented with L-glutamine (2 mM), penicillin-streptomycin (100 U/ml and 100  $\mu$ g/ml, respectively), and 10% fetal bovine serum (GIBCO Invitrogen Cell Culture). Continuous cell lines were synchronized by passaging 24 h prior to addition of reagents used for perturbations. U937 and U1 cells were seeded in

24-well plates at a concentration of  $1.0 \times 10^6$  cells/ml in a total volume of 2 ml/well.

**Reagents.** LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich, St. Louis, MO) was further purified using a triethylamine and phenol extraction protocol. Cell lines were pretreated with PMA at a final concentration of 10 nM (Sigma-Aldrich) for 24 h. Synthetic lipopeptide Pam<sub>3</sub>Cys-SKKKK x 3HCl (Pam<sub>3</sub>Cys; EMC Microcollections GmbH, Germany) was used as a specific TLR2 ligand (36). Inhibition of extracellular signal-regulated kinase (ERK) phosphorylation was performed using 2'-amino-3'-methoxyflavone, PD98059, which acts through selective and cell-permeable inhibition of upstream MAP kinase kinase (MEK; Calbiochem, San Diego, CA) (11, 25).

**Cytokine ELISAs.** Twenty-four hours after treatment with stimulus or without stimulus treatment, cell culture supernatants were collected and stored at -20°C until assayed for TNF- $\alpha$  production following the manufacturers' instructions (R&D Quantikine enzyme-linked immunosorbent assay [ELISA]; R&D Systems Inc., Minneapolis, MN).

**Flow cytometry for Toll-like receptors.** Staining was performed using phycoerythrin (PE)-conjugated anti-human TLR1, anti-human TLR4, PE-Cy5-conjugated CD11b/macrophage adhesion molecule 1 (MAC-1) and fluorescein isothiocyanate-conjugated TLR2 antibodies, or appropriate isotype control (BD and eBioscience, San Diego, CA). Following antibody incubation, cells were washed, and fixed with 4% paraformaldehyde. All incubations were performed on ice. The total volume was adjusted to 500  $\mu$ l with stain buffer, and fluorescence was detected using the FACScan machine, collected using Cell Quest program (Becton Dickinson), and analyzed using WinMDI version 2.9.

**mRNA extraction, cDNA synthesis, and real-time PCR.** RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA), following the manufacturer's instructions, with additional DNase treatment (Qiagen). RNA concentrations were determined by optical density, and 100 ng of RNA was reverse transcribed using the Advantage reverse transcription-for-PCR kit, using oligo(dT)<sub>18</sub> primer (BD Biosciences Clontech, San Jose, CA). Control RNA and primers were run in parallel with test material. cDNA (1/10 final PCR volume) was used to amplify message using the Sybr green Qiagen PCR master mix kit and an ABI 7000 machine (Foster City, CA). Cycling conditions include a hot start at 94°C for 15 min, followed by 40 to 45 cycles appropriate to primer mix. Subsequent data were exported into the Excel package (Microsoft Office) and analyzed using a semi-quantitative calculation (ABI), normalized against housekeeping gene ( $\beta$ -actin), and statistical analysis of differences was calculated.

**Western blot analysis.** Whole-cell protein extracts were prepared in 200  $\mu$ l of cell lysis buffer (0.1% sodium dodecyl sulfate, 62.5 mM Tris-HCl [pH 7.5], 10% glycerol), 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma-Aldrich). Lysates were clarified by centrifugation at 10,000 rpm in a refrigerated microcentrifuge for 15 min and quantified using the Lowry method (DC protein assay; Bio-Rad, Hercules, CA). Proteins (10 to 20  $\mu$ g/lane) were separated on a 10% NuPAGE bis-tris gel with morpholineethanesulfonic acid running buffer (Invitrogen), and transferred to nitrocellulose membrane using a Mini Trans-blot module (Bio-Rad, Hercules, CA). We detected phosphorylated ERK (p44/42) and TLR4 using eBioscience HTA125 clone or H3 as a loading control, using antibodies from Cell Signaling (Danvers, MA), following the manufacturer's guidelines. We used a chemiluminescent ECL Western blot detection system (Amersham Biosciences, Piscataway, NJ) and autoradiograms (AR film; Eastman Kodak Company, New Haven, CT). Gel images were scanned and analyzed using Total Lab image analysis software (Nonlinear Dynamics, Durham, NC). ERK and TLR4 signals were normalized against the housekeeping protein H3. Increases in phosphorylation of ERK were calculated using ratios of untreated and treated samples.

**Statistical analysis.** Data are expressed as mean values  $\pm$  standard errors of the means (SEMs). Statistical significance of differences was determined using *t* test (Student's, one tailed), Kruskal-Wallis correlation tests, and Wilcoxon two-sample tests and considered significant at *P* < 0.05.

## RESULTS

**Demographics.** The age of the subjects ranged from 27 to 51 years. At the time of evaluation, the CD4 counts for the HIV-positive subjects ranged from 70 to 917 cells/mm<sup>3</sup>, and plasma HIV RNA (viral load) ranged from  $\leq 200$  to 207,181 copies/ml as determined by reverse transcription-PCR. HIV-positive subjects were further stratified for various analyses by CD4 cell count (<200/mm<sup>3</sup> and  $\geq 200$ /mm<sup>3</sup>) and by viral load ( $\leq 10,000$  copies/ml and >10,000 copies/ml). Similar results were ob-

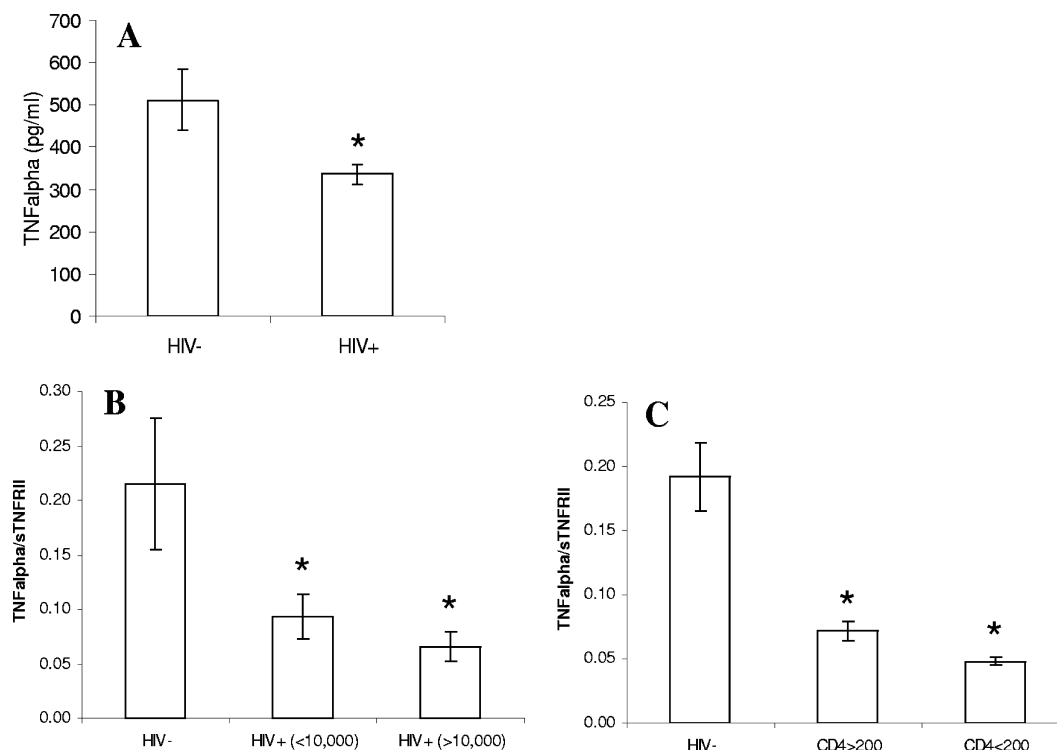


FIG. 1. (A) TNF- $\alpha$  levels in BALF samples from asymptomatic HIV-positive subjects and healthy controls. Data are means  $\pm$  SEMs (error bars) for 11 HIV-negative (HIV-) controls and 21 HIV-positive (HIV+) subjects. (B) Molar ratio of TNF- $\alpha$  to sTNFR II in BALF samples from HIV-negative controls compared to HIV-positive subjects, stratified by HIV RNA (viral load). Data are mean  $\pm$  SEMs (error bars) from 9 control subjects, 14 HIV-positive subjects with a viral load of  $\leq 10,000$  copies/ml, and 7 HIV-positive subjects with a viral load of  $> 10,000$  copies/ml. (C) Molar ratio of TNF- $\alpha$  to sTNFR II in BALF samples from HIV-negative controls compared to HIV-positive subjects, stratified by CD4 cell count (cells/mm<sup>3</sup>). Data are means  $\pm$  SEMs (error bars) from 11 HIV-negative controls, 12 HIV-positive subjects with a CD4 count of  $> 200$  cells/mm<sup>3</sup>, and 5 HIV-positive subjects with a CD4 count of  $\leq 200$  cells/mm<sup>3</sup>. Values that were significantly different ( $P = < 0.05$ , Wilcoxon two-sample test) from the value for the HIV-negative control group are indicated with an asterisk.

tained when subjects were stratified by viral load using thresholds of 400 (the lower limit of detection of the assay), 1,000, and 5,000 copies/ml. Viral loads were obtained from peripheral blood (plasma) samples. CD4 cell counts and viral load measurements were obtained within 2 to 7.5 weeks of research bronchoscopy. All HIV-positive subjects were on HAART for at least the past 8 months. At the time of research bronchoscopy, some individuals were failing therapy, as indicated by lower CD4 counts and higher viral loads. The reasons for treatment failure were not documented but did not include any obvious difficulties with adherence, since stable HAART for at least the past 8 months was an inclusion requirement of the study. Control donors were balanced for age and gender, had negative HIV antibody at the time of participation, and no evidence of pulmonary or systemic disease.

**Altered TNF- $\alpha$  levels in BALF samples from HIV-1-infected individuals.** Proinflammatory responses are important in protection against opportunistic infections, and TNF- $\alpha$  is a critical inflammatory cytokine of the innate immune system. We examined BALF fluid samples from HIV-positive individuals and found a decrease in basal TNF- $\alpha$  compared to healthy controls ( $P = 0.0135$ ; Fig. 1A). When stratified by measures of disease progression, TNF- $\alpha$  production in the lung decreased with increasing viral load and with decreasing CD4 cell counts in the blood. Moreover, sTNFR II levels in BALF samples were sig-

nificantly greater in HIV-positive individuals ( $P = < 0.05$ ) and were greater with increasing viral load and decreasing CD4 cell count. No differences in levels of sTNFR I were observed between groups (data not shown). Compared to the HIV-negative control group, this resulted in a decrease of 2.3- to 2.6-fold in the ratio of TNF- $\alpha$  agonist to sTNFR II antagonist activity in the group with less advanced HIV disease ( $\leq 10,000$  copies/ml and  $\geq 200$  cells/mm<sup>3</sup>, respectively), and a decrease of 3.2- to 4-fold in the group with more advanced HIV disease who were failing therapy ( $> 10,000$  copies/ml and  $\leq 200$  cells/mm<sup>3</sup>, respectively; Fig. 1B and C). These data show significantly altered perturbations of the inflammatory cytokine TNF- $\alpha$  and its antagonist sTNFR II in HIV-positive individuals that are associated with advancing HIV disease; these alterations in TNF- $\alpha$  and sTNFR II concentrations were noted even in subjects who had responded well to HAART, which suggests persistent defects in innate immune responses even in the group with successful anti-HIV therapy.

**HIV-1 infection impairs TLR-mediated TNF- $\alpha$  production.** To determine whether decreased basal TNF- $\alpha$  levels in BALF samples from HIV-positive patients were associated with altered TLR innate immune responses, we examined AMs from HIV-positive individuals stimulated ex vivo with either the TLR2 ligand Pam<sub>3</sub>Cys or the TLR4 ligand LPS. The secretion of TNF- $\alpha$  by AMs from HIV-positive subjects in response to



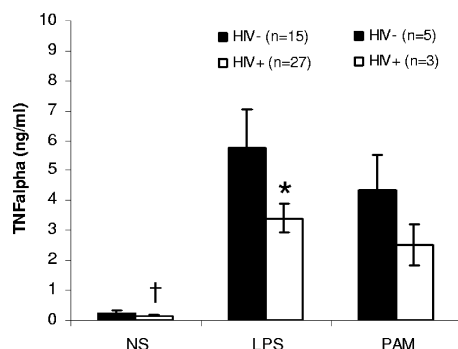


FIG. 2. TLR-induced TNF- $\alpha$  production by alveolar macrophages from HIV-negative (HIV-) healthy volunteers (solid bars) or HIV-positive (HIV+) subjects (open bars) stimulated with 100 ng/ml LPS or 10 ng/ml Pam<sub>3</sub>Cys (PAM) for 24 h. Data are means  $\pm$  SEMs (error bars). Values that were significantly different from the value for the control group (HIV-) are indicated as follows: \*,  $P = <0.05$  by  $t$  test; †,  $P = <0.05$  by Wilcoxon two-sample test. NS, no stimulation (medium alone).

both TLR ligands was decreased 1.7-fold compared with secretion by AMs from healthy controls (Fig. 2). This was statistically significant for the LPS-treated group, but not the Pam<sub>3</sub>Cys-treated group, for which fewer subject samples were available for analysis, although a similar trend was observed.

To further examine the impairment of TLR-mediated TNF- $\alpha$  production, we compared TNF- $\alpha$  production by the

chronically HIV-1-infected U1 cell line to the uninfected parental U937 cell line after stimulation with increasing doses of TLR ligand LPS or Pam<sub>3</sub>Cys for 24 h. To model AMs, U1 and U937 cells were differentiated to an AM phenotype with PMA. U937 cells had higher basal TNF- $\alpha$  concentrations and produced TNF- $\alpha$  in a dose-dependent manner in response to the TLR2 and TLR4 ligands Pam<sub>3</sub>Cys and LPS, respectively (Fig. 3A and B, solid bars); in contrast, the HIV-1-infected U1 cells produced minimal TNF- $\alpha$  in response to either TLR ligand (Fig. 3A and B, open bars). In addition, TLR-mediated TNF- $\alpha$  gene expression in the infected U1 cells was reduced compared to U937 cells, in response to either Pam<sub>3</sub>Cys (Fig. 3C) or LPS (Fig. 3D). Interestingly, there appeared to be higher basal TNF- $\alpha$  mRNA in U1 cells (no stimulation,  $P = 0.059$  [Fig. 3C] and  $P = 0.037$  [Fig. 3D] by Student's  $t$  test), although there were lower basal protein concentrations (Fig. 3A and B, no stimulation). However, the magnitude of response in the HIV-1-infected U1 cells was less than that observed in the U937 cells and appeared to be TLR-ligand specific, with an increase of fivefold in TNF- $\alpha$  mRNA in response to the TLR2 ligand Pam<sub>3</sub>Cys (Fig. 3C) and no increase in TNF- $\alpha$  gene expression in response to the TLR4 ligand LPS (Fig. 3D). In the U937 cells, there was an increase of 20-fold in TNF- $\alpha$  mRNA in response to both the TLR2 and TLR4 ligands. Moreover, this TLR-induced TNF- $\alpha$  expression was inhibited by pretreatment of cells with the ERK inhibitor PD98059, as manifested by decreased TNF- $\alpha$  mRNA and subsequent protein production

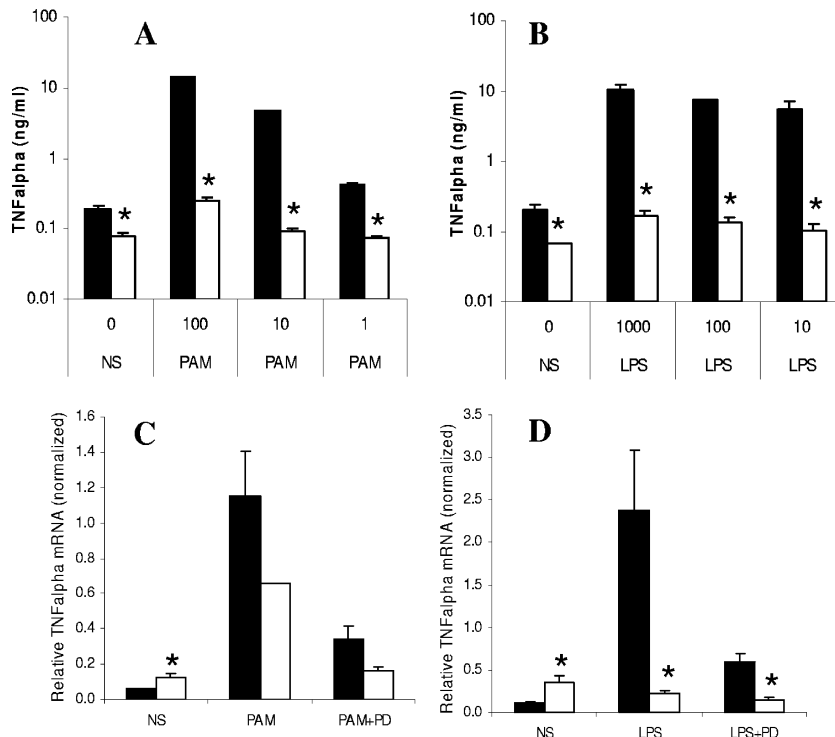


FIG. 3. TNF- $\alpha$  induction by TLR ligands in U937 cells (solid bars) and HIV-1-infected U1 cells (open bars). Cells were differentiated with 10 nM PMA for 24 h, washed, and then incubated with Pam<sub>3</sub>Cys (PAM) (A and C) or LPS (ng/ml) (B and D) for 24 h. TNF- $\alpha$  protein (A and B) was measured by ELISA, and gene expression (C and D) was measured by real-time PCR. ERK phosphorylation was blocked by 45 min pretreatment with 25  $\mu$ M PD98059 (PD) and then cells were treated with TLR ligand for 24 h. Each test was carried out in duplicate. Data are means plus SEMs (error bars) and are representative of three independent experiments. Values that were significantly different ( $P = <0.05$  by  $t$  test) from the value for the control group (HIV-) are indicated by an asterisk. NS, no stimulation (medium alone).

(protein data not shown). Taken together, the patient AM and cell line data confirm that HIV-1-infection alters the ability of TLR ligands to induce TNF- $\alpha$  production, which may result in a compromised innate immune response.

**Decreased expression of TLR1 and TLR4 on HIV-1-infected cells.** Because Pam<sub>3</sub>Cys and LPS led to reduced TNF- $\alpha$  production in AMs from HIV-positive subjects and in HIV-1-infected cells, we examined the cell surface expression of TLR1 (which forms heterodimers with TLR2), TLR2 (the receptor for Pam<sub>3</sub>Cys), and TLR4 (the receptor for LPS) on U937 and U1 cells. TLR1, TLR2, and TLR4 were present on both uninfected U937 and HIV-1-infected U1 cells; however, surface expression of TLR1 and TLR4, but not TLR2, were reduced in the U1 cells (Fig. 4B) compared to the uninfected parental cell line, U937 (Fig. 4A). We also examined TLR6, which signals with TLR2 in response to mycobacteria, and found no significant differences in expression between cell types (data not shown). Of note, TLRs were predominately detected on cells that showed an activated phenotype as determined by CD11b/MAC-1 staining. The majority of HIV-positive U1 cells (96%) were found to be CD11b/MAC-1 positive, compared to approximately 34% of the uninfected U937 cells. Both cell lines were pretreated with equal concentrations and duration of PMA; therefore, the presence of HIV is likely playing a role in the increased activation state of the U1 cells. Western blot analysis also showed decreased levels of TLR4 protein in HIV-positive U1 cells (Fig. 4C).

Reduced responses to TLR ligands could be explained by decreased surface expression of TLRs and decreased expression of TLR4 protein in the HIV-1-infected U1 cells. Consistent with these observations in a HIV-infected cell line, we also observed a downregulation of TLR gene expression in AMs from HIV-positive individuals compared to healthy controls (Fig. 4D). This difference was statistically significant for the TLR1 and TLR2 genes ( $P \leq 0.05$ ), for which a difference of 1.5- to 1.8-fold was observed. Although the difference in TLR4 gene expression was not significantly different between HIV-positive and HIV-negative healthy controls, there was a trend toward less TLR4 in the HIV-positive AMs. We did not measure TLR surface expression on AMs by fluorescence-activated cell sorting (FACS) because of insufficient cell numbers for many study subjects after other experiments were performed. These data suggest that the decreased TNF- $\alpha$  response to TLR2 and TLR4 ligands in AMs from HIV-positive subjects may be due to reduced TLR gene and receptor expression.

**Reduced phosphorylated ERK in response to TLR ligands in infected promonocytic cells.** Phosphorylation of ERK is involved in many critical signaling pathways and is required for the production of TNF- $\alpha$  protein in response to TLR ligands (25). To assess the role of ERK in TLR2 and TLR4 signaling during HIV infection, we cultured AMs and U937 and U1 cells in the presence and absence of the MAP kinase inhibitor PD98059, an upstream inhibitor of ERK phosphorylation, followed by Pam<sub>3</sub>Cys or LPS treatment. Protein extracts were analyzed by Western blotting for phosphorylated ERK. We used the H3 protein as a control, since the expression of this protein was not influenced by TLR signaling or HIV-1 infection.

When we examined cytoplasmic protein extracts from HIV-positive AMs and healthy volunteers for the presence of phos-

phorylated ERK in response to the TLR4 ligand LPS, we found decreased activation of ERK in the HIV-positive AMs compared to uninfected AMs (Fig. 5A). In cell lines, Western blot data showed an increase of two- to threefold in phosphorylated ERK in the uninfected U937 cells detected up to 60 min in response to both TLR2 and TLR4 ligands. These responses were inhibited by pretreatment of the cells with PD98059 (Fig. 5B and C). In contrast, in the infected U1 cells, increased basal levels of phosphorylated ERK were observed, but there was no significant induction of phosphorylation through 60 min in response to either Pam<sub>3</sub>Cys (Fig. 5B) or LPS (Fig. 5C). U1 cells showed minimal activation of ERK in response to either ligand, and treatment with the inhibitor did not significantly decrease phosphorylation of ERK. Therefore, unlike U937 cells, the HIV-1-infected U1 cells did not induce further signaling upon treatment with TLR ligands. In U1 cells, there were higher basal levels of ERK, which was associated with dampened responses to TLR2 and TLR4 ligands; this may in part explain decreased TNF- $\alpha$  production. Therefore, altered signaling through ERK in the HIV-1-infected cell lines and AMs from HIV-positive subjects was associated with decreased inflammatory cytokine production in response to LPS and Pam<sub>3</sub>Cys.

## DISCUSSION

Most studies of host immunity against HIV and opportunistic pathogens that affect HIV-positive individuals have focused on the adaptive immune response. Less is known about the more primitive innate immune response during HIV infection. In this study, we examined BALF samples and alveolar macrophages from HIV-positive subjects and healthy matched HIV-negative control subjects. We extended our investigation using the PMA-differentiated monocyte cell line U937 (23) and its HIV-1-infected subclone U1 to mimic an AM phenotype. We determined cell membrane expression of TLR1, TLR2, and TLR4 and found decreased surface expression of TLR1 and TLR4 on the HIV-1-infected U1 cell line as well as decreased TLR4 protein compared to the uninfected U937 cells. Due to low cell numbers obtained from bronchoalveolar lavage in this study group, we were unable to perform FACS for TLR surface expression on AMs, although results from real-time PCR showed decreased gene expression of a subset of TLRs (Fig. 4D). We also found significantly less production of TNF- $\alpha$  in response to TLR2 and TLR4 ligands in U1 cells and in AMs from HIV-positive subjects (TLR4 ligand) and a trend toward decreased responses to the TLR2 ligand in AMs from HIV-positive subjects, for which fewer samples from study subjects were available for analysis (Fig. 2).

Studies have shown that HIV infection quickly depletes intestinal lymphoid tissues and compromises mucosal integrity (6). This leads to "microbial translocation" and the presence of bacterial products in the peripheral circulation. These bacterial products include LPS from gram-negative bacteria and peptidoglycan, the naturally occurring TLR2 ligand analog of Pam<sub>3</sub>Cys from gram-positive bacteria that colonize the normal human intestine. Thus, the TLR ligand responses we studied have direct relevance to persons with HIV infection. Brechley (5) and others have shown that plasma levels of LPS correlate with chronic immune activation of CD4 cells; this may lead to

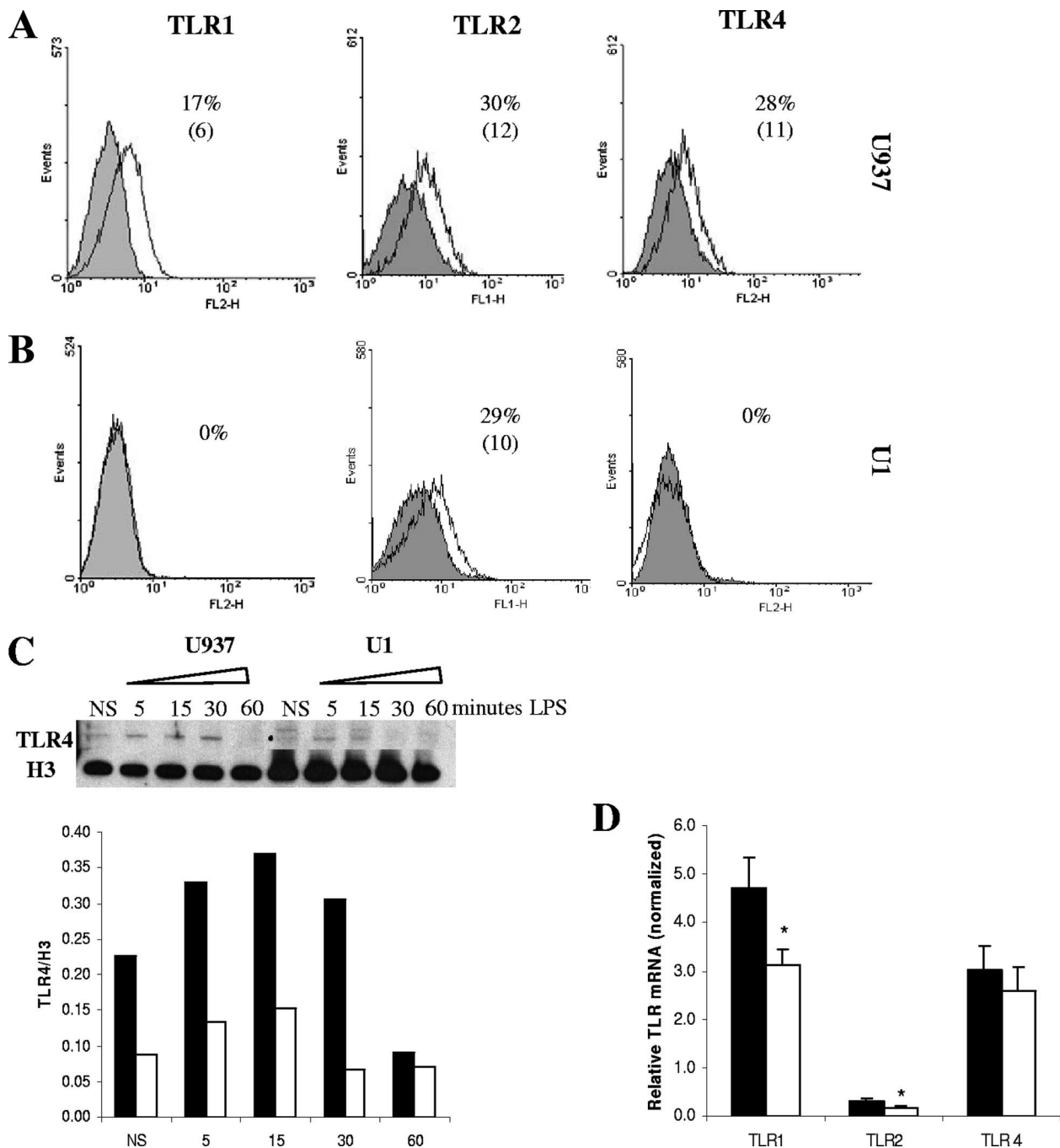


FIG. 4. Flow cytometric analysis of TLR1, TLR2, and TLR4 constitutive surface expression on PMA-pretreated (10 nM for 48 h) U937 (A) and U1 (B) cells. Cells were labeled with PE-Cy5-conjugated CD11b/MAC-1 antibody, PE-conjugated TLR1 (left), fluorescein isothiocyanate-conjugated TLR2 (middle), or PE-conjugated TLR4-PE (right) antibody or matched isotype control. Cells were gated on CD11b/MAC-1-positive cells. Thirty-four percent of U937 cells and 97% of HIV-positive U1 cells were CD11b/MAC-1 positive. Representative histograms show isotype (shaded) or test antibody (black line). The percentage of positive cells for test antibody and the geometric mean fluorescence intensity in parentheses are shown for each graph in panels A and B. Samples were collected by FACS and analyzed with WinMDI version 2.9 software. (C) Whole-cell protein extracts from cell lines pretreated with PMA were analyzed by Western blotting for the presence of TLR4 and control protein H3. We used densitometry and the Total Lab computer package to determine band intensities and normalized the TLR4 band with control H3 for no stimulation (NS) and LPS treatment (in minutes) for U937 (solid bars) and U1 (open bars) cells. The height of the triangle above the gel shows the increasing amount of time after LPS treatment. (D) Total RNA from HIV-negative healthy volunteers ( $n = 4$ ; solid bars) and HIV-positive ( $n = 5$ ; open bars) alveolar macrophages was used to perform two-step real-time PCR analysis. Data are mean relative mRNAs plus SEMs (error bars), normalized using the  $\beta$ -actin housekeeping gene. Values for HIV-positive AMs that were significantly different from the value for HIV-negative AMs are indicated as follows: \*,  $P = <0.05$  by  $t$  test.

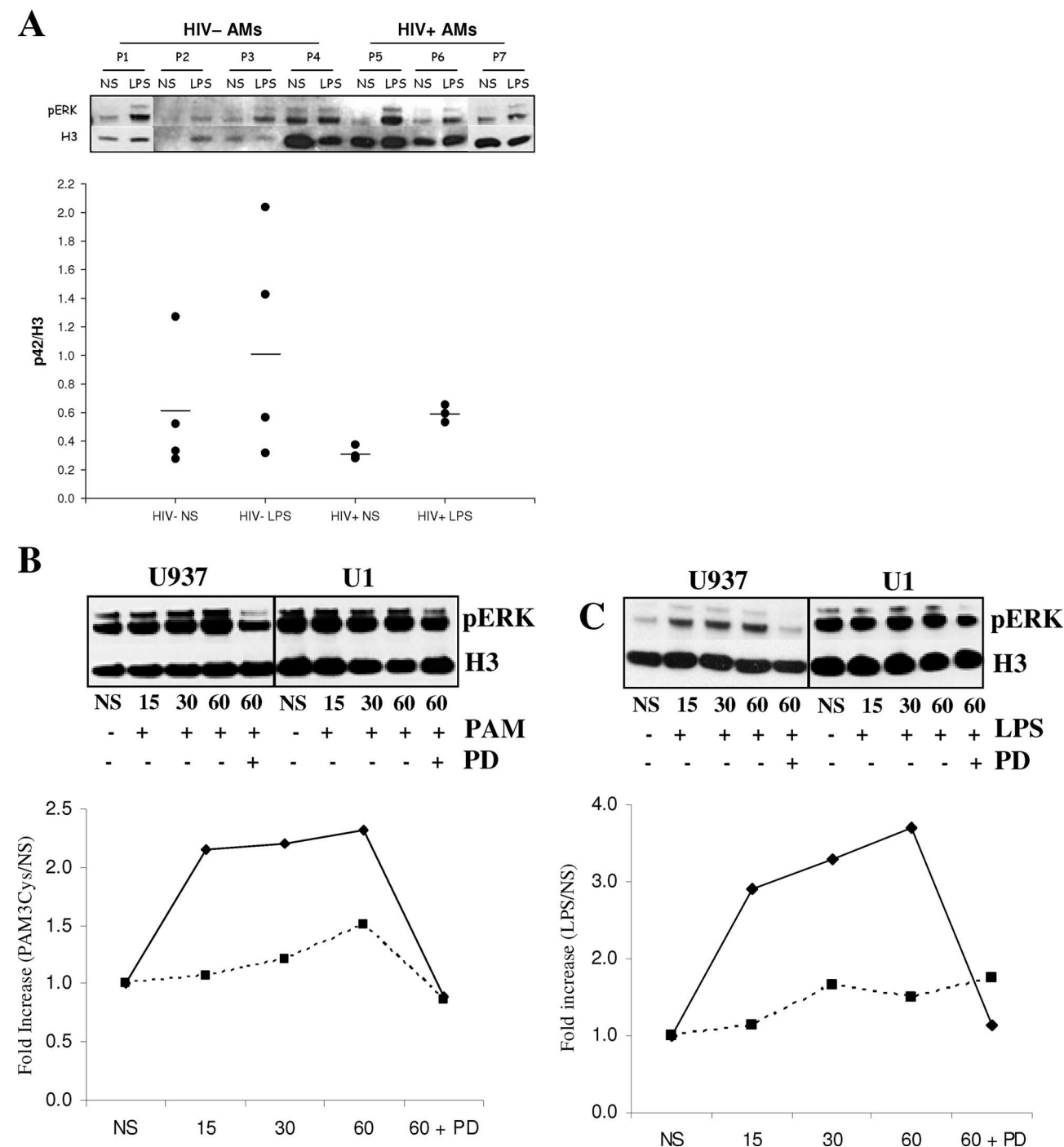


FIG. 5. (A) Cytoplasmic protein extracts from HIV-positive (HIV+) AMs ( $n = 3$ ; patient 5 [P5] to P7) and HIV-negative (HIV-) healthy volunteers ( $n = 4$ ; P1 to P4) were analyzed by Western blotting for the presence of phosphorylated ERK (pERK) (p42/44) and control protein H3. We used densitometry and the Total Lab computer package to determine band intensities and normalized the phosphorylated ERK p42 band with control H3 (shown in graph) for no stimulation (NS) and LPS treatment. (B and C) Differentiated U937 cells (solid line) and U1 cells (dashed line) were treated with (+) either Pam<sub>3</sub>Cys (PAM) (10 ng/ml) (B) or LPS (100 ng/ml) (C) for 15, 30, or 60 min with (+) or without (-) 30 min pretreatment with the ERK inhibitor PD98059 (PD) (25  $\mu$ M). We analyzed Western blots on whole-cell lysates by densitometry, and increases in phosphorylation of ERK were determined from ratios of normalized treatment over no stimulation (NS).

T-cell activation, increased entry into the cell cycle, and ultimately increased death of central memory CD4 cells, which is one of the hallmarks of the immunodeficiency associated with HIV infection (N. T. Funderburg, A. Luciano, W. Jiang, S.

Sieg, and M. Lederman, presented at the Fourth International Conference on HIV Pathogenesis, Treatment, and Prevention, Sydney, Australia, 22 to 25 July 2007). Our data concerning the effects of LPS on innate immune function through TLR4 in-



dicating that similar mechanisms may be pertinent in vivo through microbial translocation.

Viral infection can cause alterations in TLR4 protein expression and membrane localization, altering the lung environment and innate immune responses, with a subsequent switch from an LPS-tolerant state to a LPS-responsive state (4, 22, 27, 34). Several HIV-1 accessory proteins have been implicated in the regulation of TLR expression and signaling (24). HIV-1 Nef protein altered TLR-dependent cytokine production in AMs from HIV-positive subjects through induction of cellular phosphatases and inhibition of intracellular signaling (35). HIV may utilize multiple strategies to alter TLR expression and signaling, and these effects may vary based on cell type and location. Heggelund et al. (14) showed enhanced expression of TLR2 (but not TLR4) on peripheral blood monocytes from HIV-positive subjects after exposure to the HIV-1 envelope protein gp120. Effects on TLR expression may vary in relation to the macrophage type (from different body compartments); specifically, AMs may differ from peripheral blood monocytes in terms of TLR expression during HIV infection. Reports of the expression of TLRs on U1 cells have also varied (29, 35), perhaps due to differences in experimental procedures and conditions, such as the concentration of the PMA used or the cell passage number. We have also shown that more U1 cells become activated, as measured by CD11b/MAC-1 expression, than U937 cells after PMA stimulation, and that the majority of TLR expression occurs on these activated cells. Further studies to determine the role of macrophage maturation on TLR expression and signaling will be of interest.

TNF- $\alpha$  is a cytokine which has pleiotropic biological actions, and since cytokines act locally, examining concentrations in specific organs, such as the lung, is important. The balance of TNF- $\alpha$  with antagonist moieties, such as sTNFR<sub>II</sub>, determines, in part, net agonist activity of TNF- $\alpha$ , since sTNFR<sub>II</sub> acts as a "scavenger" for TNF- $\alpha$ . The balance of TNF- $\alpha$  and sTNFR<sub>II</sub> in the lung likely determines innate immune function, rather than plasma concentrations as measured in other studies (15, 16, 31). In our study, the ratio of TNF- $\alpha$  to sTNFR<sub>II</sub> was lowest in the groups with the highest viral load and lowest CD4 cell count; higher ratios indicate more abundant agonist TNF- $\alpha$  activity, which we postulate is associated with normal innate immune function in the lung. Anti-TNF- $\alpha$  therapies highlight the importance of TNF- $\alpha$  in normal host innate immunity, since their use in Crohn's disease (18) and rheumatoid arthritis (21) is associated with an increased incidence of mycobacterial and other bacterial infections, especially in the lung (32, 37).

Although sTNFR<sub>II</sub> "scavenges" soluble TNF- $\alpha$  and limits binding to surface receptors, thereby acting as an inhibitor, it has also been shown to stabilize TNF- $\alpha$ , preserving its activity and potentially augmenting longer-term effects (2). It may be that stabilizing TNF- $\alpha$  in compartments, such as alveolar spaces, where the clearance of TNF- $\alpha$  is already slow (28), may have significant biological consequences. The absolute amounts of TNF- $\alpha$  in BALF may not be as significant as the relationship between sTNFR<sub>II</sub> and its stability in the lung environment, which could lead to persistently abnormal innate immune function.

Cytokines also play a role in TLR signaling through alterations in expression of TLRs. TNF- $\alpha$  and IFN- $\gamma$  regulate TLR4 expression in intestinal epithelial cells (1) and TLR4 in

monocytes and macrophages (3). This makes biologic sense, since tight control of inflammatory responses at mucosal surfaces, which are exposed to commensal bacteria and exogenous agents, would be beneficial to host defense. In addition to decreased TNF- $\alpha$  production by AMs exposed to Pam<sub>3</sub>Cys or LPS, we also observed decreased responses in other pathways involved in TLR-induced TNF- $\alpha$  production, such as NF- $\kappa$ B p50 nuclear translocation (data not shown) and TNF- $\alpha$  gene expression. Moreover, there was decreased phosphorylation of ERK in AMs from HIV-positive subjects and infected cell lines. It is interesting to note there were increased basal (constitutive) levels of phosphorylated ERK in the U1 cell line and subsequent lower increases in response to TLR ligands, which may indicate that chronic stimulation of TLRs may "tolerize" their receptors and lead to defective TLR signaling and innate immune responses. This concept is supported by the observation of deficits in TLR-dependent TNF- $\alpha$  production in U1 cells that were almost all in the activated state as measured by CD11b/MAC-1 staining (Fig. 3). Further studies will be required to determine whether decreased phosphorylation of other MAP kinases, such as p38 and Jun N-terminal protein kinase, in response to HIV infection, is also evident.

Taken together, our studies show altered TLR-dependent innate immune responses of AMs from HIV-positive subjects and in HIV-1-infected cell lines. In future studies, it will be important to determine the effects of other TLR ligands on innate immune responses and whether altered surface receptor expression involves changes in transcription, translation, or membrane transport of TLRs. Our findings of altered pathogen recognition receptors and lower innate immune responses, through indirect cytokine or direct viral protein effects, may help explain the increased occurrence of OIs and other pulmonary infections in HIV-positive individuals.

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